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Secretion and *in vivo* folding of the F_{ab} fragment of the antibody McPC603 in *Escherichia coli*: influence of disulphides and *cis*-prolines

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Using the well-characterized antibody McPC603 as a model, we had found that the F_v fragment can be isolated from *Escherichia coli* as a functional protein in good yields, whereas the amount of the correctly folded F_{ab} fragment of the same antibody produced under identical conditions is significantly lower. In this paper, we analyse the reasons for this difference. We found that a variety of signal sequences function in the secretion of the isolated chains of the F_{ab} fragment or in the co-secretion of both chains in *E.coli*. The low yield of functional F_{ab} fragment is not caused by inefficient expression or secretion in *E.coli*, but by inefficient folding and/or assembly in the periplasm. We compared the folding yields for the F_v and the F_{ab} fragment in the periplasm under various conditions. Several diagnostic framework variants were constructed and their folding yields measured. The results show that substitutions affecting *cis*-proline residues and those affecting various disulphide bonds in the protein are by themselves insufficient to dramatically change the partitioning of the folding pathway to the native structure, and the cause must lie in a facile aggregation of folding intermediates common to all structural variants. However, all structural variants could be obtained in native form, demonstrating the general utility of the secretory expression strategy.

Key words: antibody/expression in *E.coli*/protein folding/protein secretion

Introduction

The development of methods for the expression of functional antigen-binding fragments of antibodies in *Escherichia coli* (Better *et al.*, 1988; Skerra and Plückthun, 1988; Plückthun and Skerra, 1989) paved the way for the facile and rapid access to engineered antibodies. The expression strategy for secreting both chains of the antibody fragment in the same cell is suitable for both F_v fragments (Skerra and Plückthun, 1988) and F_{ab} fragments (Better *et al.*, 1988; Plückthun and Skerra, 1989). Furthermore, both chains of the F_v fragment can be linked to generate a secreted single-chain antibody fragment (Glockshuber *et al.*, 1990) with almost unaltered binding affinity. Recently, the expression technology was extended to the expression of libraries of antibody fragments in *E.coli* (Huse *et al.*, 1989; Ward *et al.*, 1989).

The model antibody with which the studies described here were carried out is the mouse IgA McPC603, which binds the hapten phosphorylcholine. The amino acid sequence of this myeloma protein (Rudikoff and Potter, 1974; Rudikoff *et al.*, 1981) and its binding properties (Leon and Young, 1971; Metzger *et al.*, 1971; Young and Leon, 1977) are well documented and, most importantly, the three-dimensional structure of the F_{ab} fragment in the presence and absence of phosphorylcholine is known (Segal *et al.*, 1974; Satow *et al.*, 1986). Previous studies on the F_v fragment of the antibody McPC603 (Skerra and Plückthun, 1988; Glockshuber *et al.*, 1990), which consists only of the V_L and the V_H domains, showed that in the absence of the hapten the two chains dissociated upon dilution. This phenomenon leads to a limited stability of the uncomplexed F_v fragment, which is particularly pronounced at physiological temperature. The association of the two domains can be improved either by covalently linking the chains (Glockshuber *et al.*, 1990) or by using the F_{ab} fragment, which in addition to the variable domains V_L and V_H also contains the constant domains C_L and C_H1. Preliminary studies had shown, however, that the amount of functional F_{ab} fragment isolated from *E.coli* is significantly smaller than the amount of functional F_v fragment, although the total amounts of protein synthesized are rather similar in both cases. In this paper, we analyse this phenomenon in more detail and describe investigations of the potential reasons for this different folding behaviour of the two antibody fragments.

While we cannot rule out that antibodies of different sequence and specificity may have folding bottlenecks at different kinetic steps, we wished to test the effects of structural changes in the framework with one set of well-characterized mutants of an antibody with known structure. We report here the effects of mutations affecting *cis*-prolines, disulphide bonds and the nature of the antibody class on antibody folding in *E.coli*, using the antibody McPC603 as a model.

Materials and methods

Materials and strains

Plasmids pASK30 (Skerra *et al.*, 1991) and pAP5 (Laminet and Plückthun, 1989) were from our own collection. The pUC series plasmids used (Vieira and Messing, 1982; Yanisch-Perron *et al.*, 1985) were purchased from Pharmacia. pINIII-ompA1 (Ghrayeb *et al.*, 1984) was obtained from M.Inouye, and pHI61 with the promoter and 5′-terminal region of the alkaline phosphatase gene (Inouye *et al.*, 1982) was obtained from H.Inouye. *Escherichia coli* strain JM83 [*ara*, Δ(*lac-pro*AB) *rps*L, ϕ80, *lac*ZΔM15] (Yanisch-Perron *et al.*, 1985) was from our collection. JA221 [*lpp*$^-$, *hsd*R$^-$, Δ*trp*E5, *leu*B6, *lac*Y, *rec*A1 (F′:*lac*I^q, *lac*$^+$, *pro*$^+$] (Takahara *et al.*, 1985) was from M.Inouye, and the wild-type strain *E.coli* B (ATCC 11303) was from the American Type Culture Collection. Strain SM547 (MC1000, *pho*R Δ(*pho*A-*pro*C) *tsx*::Tn5) was from J.Beckwith.

General methods

All DNA manipulations were carried out using standard methodology (Maniatis *et al.*, 1982). The genes encoding for the mutant F_{ab} fragments *H136-143, H22,98, H198,222* and *L220* were obtained by site-directed mutagenesis (Geisselsoder *et al.*,

1987; Kunkel *et al.*, 1987; Vieira and Messing, 1987; Skerra, 1989; Skerra *et al.*, 1991) with single-stranded DNA derived from the plasmid vector pASK29 (see below) or its derivatives.

Expression vectors

The construction of the genes encoding both chains of the McPC603 F_{ab} fragment was described earlier (Plückthun *et al.*, 1987). The effect of the choice of the signal sequence on the secretion of both chains of the McPC603 F_{ab} fragment in *E.coli* was investigated with the plasmids shown schematically in Figure 1. They encode fusions with the signal sequences of the *E.coli* proteins β-lactamase (pASK5 and pASK6), alkaline phosphatase (pASK7 and pASK8) and outer membrane protein A (pASK13 and pASK14) (Skerra, 1989). The plasmids pASK5, pASK7 and pASK13 (Figure 1) encode precise fusions of these signal sequences with the light chain of the F_{ab} fragment (κ chain), whereas the vectors pASK6, pASK8 and pASK14 code for the corresponding fusions with the heavy chain of the F_{ab} fragment (the so-called F_d fragment).

In the plasmids pASK5 and pASK6, which were derived from pAP5 (Laminet and Plückthun, 1989), the preproteins of the two chains of the F_{ab} fragment with the β-lactamase signal sequence are under the control of the *tac* promoter (Amann *et al.*, 1983). pASK7 and pASK8 carry the promoter–operator region and the 5′ region of the alkaline phosphatase gene including its signal sequence (Inouye *et al.*, 1982), which was precisely fused to either of the two immunoglobulin genes. These plasmids carry the replication origin and the ampicillin-resistance gene of the pUC plasmids (Vieira and Messing, 1982). pASK13 and pASK14 are direct derivatives of the pUC plasmids in which a gene fragment coding for the outer membrane protein A (OmpA) signal sequence (Ghrayeb *et al.*, 1984) was placed under control of the plasmid-encoded *lac* promoter (Skerra *et al.*, 1991).

The expression plasmid pASK11 codes for the heavy chain of the McPC603 F_{ab} fragment with the presumed original N-terminal signal peptide of the myeloma protein. While the signal sequence of the heavy chain of McPC603 has not been directly determined, it is derived from the same germ-line gene (called V_{T15} or V1) as the antibody MOPC167, of which the signal sequence is known (Kim *et al.*, 1981). Thus, it is to be expected that both signal sequences are identical. Except for the coding region of the signal sequence, pASK11 is identical to pASK14.

The functional expression of the McPC603 F_{ab} fragment was achieved by co-expression and co-secretion of both chains (Plückthun and Skerra, 1989). For studying its expression and that of mutant fragments, the expression plasmids pASK29 and pASK37 (Figure 1) were used. These plasmids encode both chains of the F_{ab} fragment arranged in an artificial operon under transcriptional control of the *lac* promoter–operator. In this case, the heavy chain carries the *omp*A signal sequence and the light chain is fused to the *pho*A signal sequence. Both chains are simultaneously expressed upon isopropyl-β-D-thio-galactopyranoside (IPTG) induction. The vector pASK29 is identical to pASK30 (Skerra *et al.*, 1991), but encodes the C_L and C_H1 domain in addition to the variable domains. pASK37 differs from pASK29 in so far as it carries the UV5 derivative of the *lac* promoter, which was derived from the plasmid pINIII-ompA1 (Ghrayeb *et al.*, 1984).

The heavy chain for the *IgG1* variant of the McPC603 F_{ab} fragment was constructed (Skerra, 1989) by replacing the C_H1 α gene fragment by the corresponding C_H1 γ1 fragment from the plasmid pIgH2 (Honjo *et al.*, 1979) in pASK29. During the resequencing of the cloned gene fragment a discrepancy to the published nucleotide sequence was found. The nucleotides 318–321 (as numbered in Figure 6 of Honjo *et al.*, 1979) read 'ACCT' instead of 'CCTC' as reported by Honjo *et al.* (1979), thus encoding the amino acid residues ThrH196 and TrpH197 instead of Pro and Arg [numbers given for the F_{ab} fragment of McPC603 in simple consecutive numbering, corresponding to positions 198 and 199 in the consensus numbering of Kabat *et al.* (1987)]. It should be noted that in all other mouse IgG subclasses the residues at this position are also Thr and Trp. The same sequence discrepancy has been noted independently (Buckel *et al.*, 1987).

Expression and purification of the antibody fragments from E.coli

The preparative purification of functional F_{ab} and F_v fragments from *E.coli* was essentially performed as previously described (Skerra and Plückthun, 1988). Cultures of *E.coli* strain JM83 harbouring the plasmid pASK30, pASK29 or pASK37 (or its derivatives coding for the mutant F_{ab} fragments) were grown in 2 l of LB-medium (Maniatis *et al.*, 1982) with 100 μg/ml ampicillin. Expression was induced by the addition of 1 mM IPTG (final concentration) at an OD_{550} of a 0.5. After induction for 45 min (for cultures grown at 37°C) or 3 h (for cultures grown at ambient temperature), the cells were harvested by centrifugation at 4000 *g* for 10 min (4°C). Under these conditions, the outer membrane of the *E.coli* cells is still intact, and there is practically no loss of the expressed protein by leakage from the periplasmic space to the culture medium (Plückthun and Skerra, 1989). Unless noted, all subsequent steps were carried out at 4°C. In order to minimize errors in the quantification of expression yields that might be caused by the cell fractionation procedures, the cells were resuspended in 15 ml BBS buffer (200 mM borate/NaOH, pH 8.0, 160 mM NaCl) and homogenized in a French pressure cell (Aminco SLM Instruments). The turbid total cell extract thus obtained was cleared by centrifugation at 48 000 *g* for 30 min. After passage through a sterilizing filter 10 ml of the resulting solution was directly applied to a phosphorylcholine affinity column. The column was washed with BBS buffer, and pure antibody fragment was eluted with a solution of 1 mM phosphorylcholine in BBS buffer.

For comparing the secretion of the two chains of the F_{ab} fragment linked to different signal sequences, the *E.coli* expression was performed on an analytical scale using cultures of 10 or 20 ml LB medium. In the case of the plasmids pASK5 and pASK6, the wild-type strain *E.coli* B was used as host, whereas the expression with the plasmids pASK11, pASK13 and pASK14 was analysed with the *E.coli* strain JA221. The cells were typically grown to an OD_{550} of 0.5 at 37°C and expression was induced by addition of 1 mM IPTG (final concentration) for 1 h. In the case of the plasmids pASK7 and pASK8, the expression was studied under constitutive conditions using the *pho*R^- strain SM547 after growing the cells to a final OD_{550} of 0.5 at 37°C.

Cell fractionation

Cell fractionations were performed according to a modification of the procedure of Witholt *et al.* (1976). After centrifuging the cells at 4600 *g* for 5 min, the cell pellet was resupended in 200 μl spheroplast buffer (200 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 0.5 M sucrose) at 4°C. Eighty microlitres of a solution of 10 mg/ml lysozyme in the same buffer were added, and after mixing, 720 μl spheroplast buffer, diluted with 1 vol water, was immediately added. The suspension was gently mixed, incubated for 30 min on ice, and finally centrifuged in a microcentrifuge

for 5 min at 4°C. The supernatant was separated as the periplasmic fraction, whereas the pellet was directly dissolved in SDS–PAGE loading buffer as the spheroplast fraction.

Protein–gel electrophoresis and immunoblotting

SDS–PAGE was performed using the discontinuous system of Fling and Gregerson (1986). To analyse the proteins with intact disulphide bonds, 2-mercaptoethanol was omitted from the loading buffer. Bands were visualized by silver staining ('Quick silver' staining kit, Amersham).

Immunoblotting was essentially performed according to Blake *et al.* (1984). The antiserum used was obtained from rabbits immunized either with the intact antibody McPC603 purified from mouse ascites or with the proteolytically prepared F_{ab}' fragment of this antibody (Plückthun *et al.*, 1987; Skerra *et al.*, 1990). The immunoreactive bands were detected with pig anti-rabbit immunoglobulin conjugated with alkaline phosphatase (Dakopatts).

Results

Functional yield and promoter strength

To investigate whether the yield of functional F_{ab} fragment produced by *E.coli* depends on the strength of the promoter, the F_{ab} fragment was expressed under the control of either the *lac* wild-type or the ~3-fold stronger *lac*UV5 promoter (Dickson *et al.*, 1977). The expression of the F_{ab} fragment was also compared with the F_v fragment. These experiments were carried out both with cells grown at 37°C and cells grown at ambient temperature (23°C).

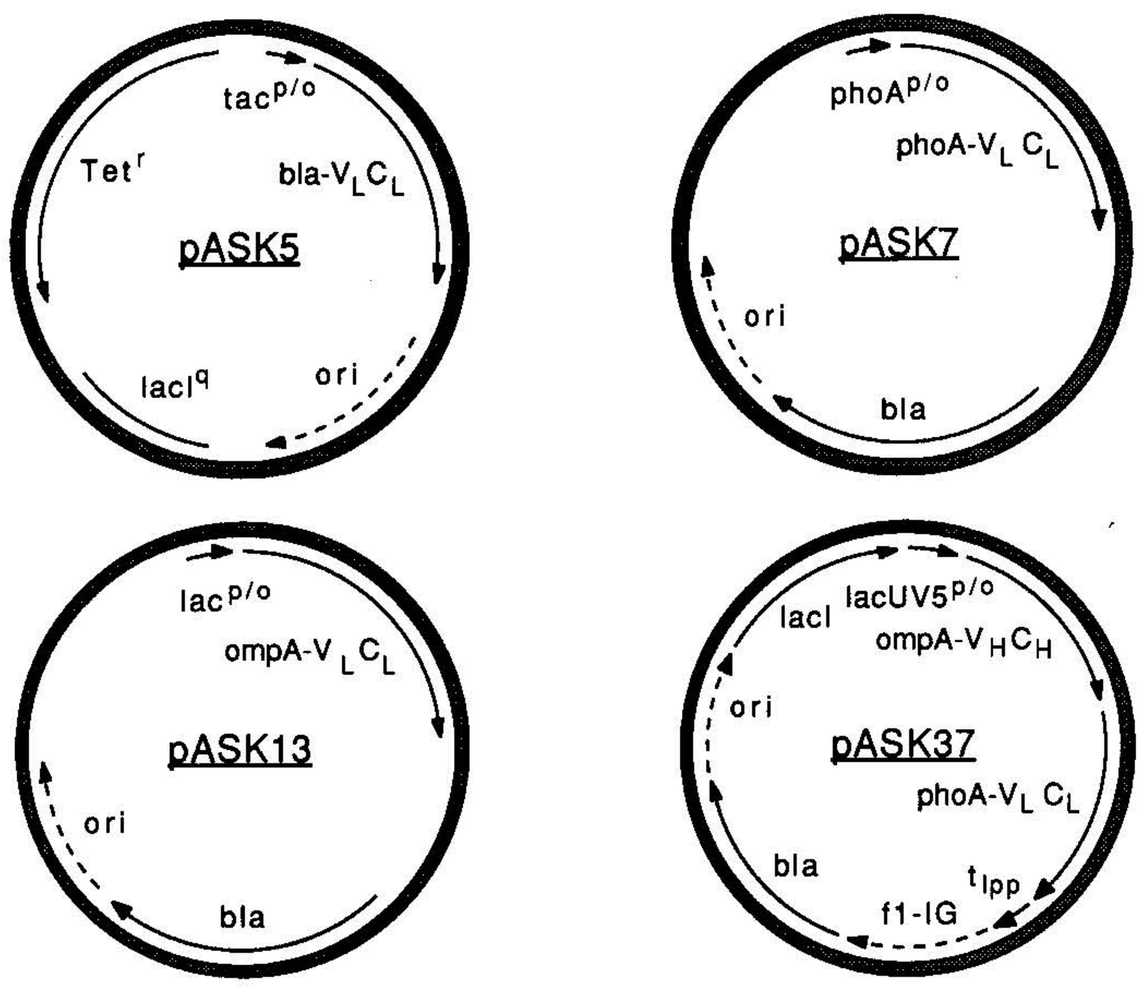

Fig. 1. Schematic drawing of the expression vectors pASK5, pASK7, pASK13 and pASK37 (see text).

As shown in Table I, the stronger promoter does not give rise to a higher yield of functional F_{ab} fragment. Rather, the yield stays approximately constant. This is found despite the fact that the amount of total recombinant protein detected (i.e. both soluble and insoluble) is significantly larger with the stronger promoter at both growth temperatures. This result was obtained by comparing the amount of F_{ab} fragment present in the total cell protein with a dilution series of purified F_{ab} fragment by immunostaining (Figure 2). Therefore, the relative proportion of functional recombinant F_{ab} fragment produced by *E.coli* actually decreases with increase of the promoter strength.

While with the stronger *lac*UV5 promoter an immunoreactive band is observed that might be a precursor for one of the chains of the F_{ab} fragment (Figure 2), no such band is seen with the wild-type *lac* promoter. The low yield of functional protein is therefore not simply a consequence of inefficient transport and processing. Thus, the limiting factor must involve the folding and assembly of the F_{ab} fragment.

In this respect the effect of the growth temperature of the *E.coli* cells on the yields of the antibody fragments is worth mentioning (Table I). The functional yield of recombinant antibody fragment is consistently higher upon expression at room temperature than at 37°C, an effect that has already been observed for a number of other proteins (see, for example, Takagi *et al.*, 1988). Interestingly, however, this phenomenon is more pronounced for the F_v fragment than for the F_{ab} fragment of McPC603.

Role of the signal sequences

In order to elucidate the effect of the signal sequences on the expression behaviour of the F_{ab} fragment in *E.coli*, we investigated the secretion of the individual chains (F_d and $\varkappa$, corresponding to the domain structures V_HC_H and V_LC_L respectively) of this antibody fragment. Three different signal sequences were employed for each chain, those of the periplasmic *E.coli* proteins β-lactamase (*bla*) and alkaline phosphatase (*pho*A), and that of the outer membrane protein A (*omp*A). As shown in Figure 3, processing of the chains was observed in all cases.

To verify that this result really indicates secretion to the periplasm, cell fractionations were carried out. The results for the *pho*A signal are shown in Figure 4. As can be deduced from the Western blot, the light chain is present both in the periplasmic fraction and in the spheroplast fraction. The heavy chain, on the other hand, cannot be detected in the soluble periplasmic fraction at all. It quantitatively co-sediments with the spheroplast fraction.

Table I. Yield of F_v and F_{ab} fragments at different growth temperatures and under the control of different promoters

Fragment	Strain/plasmid	Promoter	Growth temperature	OD_{550} at harvest	Total yield[a]	Functional yield[b]	%[c]
F_v	JM83/pASK30	*lac*	37°C	1.08	0.5	0.074	15
F_v	JM83/pASK30	*lac*	Ambient	1.68	1.0	0.55	55
F_{ab}	JM83/pASK29	*lac*	37°C	1.08	0.6	0.03	5
F_{ab}	JM83/pASK29	*lac*	Ambient	1.44	2.5	0.17	7
F_{ab}	JM83/pASK37	*lac*UV5	37°C	1.04	10	0.051	0.5
F_{ab}	JM83/pASK37	*lac*UV5	Ambient	1.39	10	0.13	1.3

[a]The total yield as estimated from Western blots is given in mg/l/OD_{550}.
[b]The yield of functional F_{ab} fragment as estimated from the amount isolated by affinity chromatography is given in mg/l/OD_{550}.
[c]The percentage of functional F_{ab} fragment is calculated from the ratio of functional protein to totally expressed F_{ab} fragment.

A similar behaviour of both chains was observed with the *bla* and *omp*A signal sequences (data not shown).

The co-sedimentation of the mature F_d fragment with the spheroplast fraction is most likely an indication of the heavy chain being secreted, processed, but precipitating in the periplasm. The following arguments support this interpretation. (i) The heavy chain, when secreted alone, has the same mol. wt as in the recombinant F_{ab} fragment isolated in a fully functional form by hapten-affinity chromatography. All signal sequences give rise to the same size fragment. It also has the same size as the F_d fragment expressed without signal sequence (Plückthun *et al.*, 1987). The F_d fragment is thus processed, even when secreted alone. (ii) Isolated F_d chains are generally poorly soluble *in vitro* at neutral or basic pH (summarized by Nisonoff *et al.*, 1975). (iii) With the same signal sequence, functional F_{ab} fragment can be obtained when the light chain is co-secreted in the same *E.coli* cell (though only a fraction of the F_d fragment associates with the light chain).

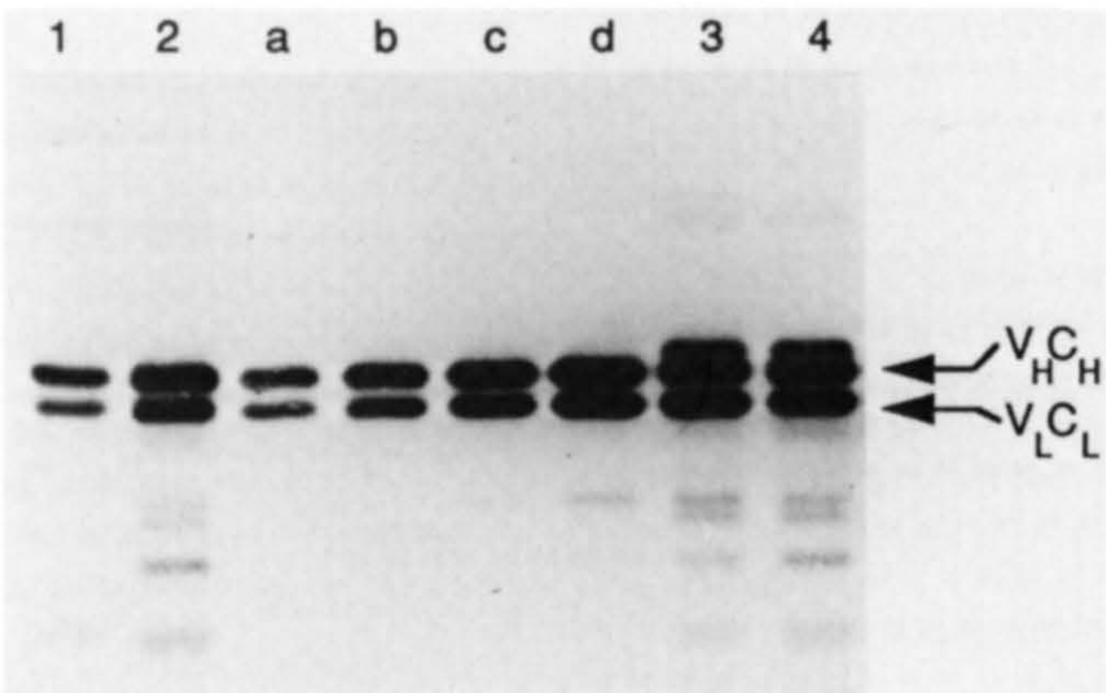

Fig. 2. Comparison of the total amount of F_{ab} fragment made in JM83/pASK29 (*lac* promoter, **lanes 1** and **2**) and pASK37 (*lac*UV5 promoter, **lanes 3** and **4**). An aliquot of total protein of cells grown at 37°C (lanes 1 and 3) or room temperature (lanes 2 and 4) was separated on a 12.5% polyacrylamide gel, transferred to nitrocellulose and detected by immunostaining. For the plasmid containing the *lac* promoter, 0.4 ml·OD_{550} was applied to the gel, whereas 0.2 ml·OD_{550} was applied for the plasmid containing the *lac*UV5 promoter. Purified F_{ab} fragment was used as the internal standard (**lane a**, 0.25 μg; **lane b**, 0.5 μg; **lane c**, 1.0 μg and **lane d**, 2.0 μg).

In order to clarify whether the observed distribution of the recombinant F_d fragment between the *E.coli* cell fractions is an effect caused by the bacterial signal sequences employed, the secretion of this chain with its original eukaryotic signal peptide was also investigated. Interestingly, the expression of the F_d fragment did not lead to a correctly secreted product with the expression plasmid pASK11. Immediately after induction of expression by addition of IPTG (either at OD_{550} of 0.5 or 1.0) growth of the cells stopped, but no lysis occurred. No protein was detected in either the periplasmic or spheroplast fraction (data not shown). Apparently, this preprotein is toxic for the *E.coli* cells.

In conclusion, both chains of the F_{ab} fragment can be secreted, either alone or in concert, but the heavy chain is particularly prone to precipitation. This is observed both when it is secreted alone as well as when it is secreted together with the light chain. It is likely, therefore, that structural reasons for inefficient folding of the F_{ab} fragment must at least in part reside in the F_d chain.

Structural factors for assembly of the F_{ab} fragment

The rationale for the investigation of structural factors influencing the folding behaviour of the F_{ab} fragment was as follows. According to the present knowledge, the transported protein emerges from the cytoplasmic membrane in a folding state distinct from native and with its disulphide bonds not yet formed (Pollitt and Zalkin, 1983; Eilers and Schatz, 1986; Randall and Hardy, 1986). Periplasmic folding, including the oxidative formation of the disulphide bonds, must proceed from this state through folding intermediates to a native-like structure that is able to associate

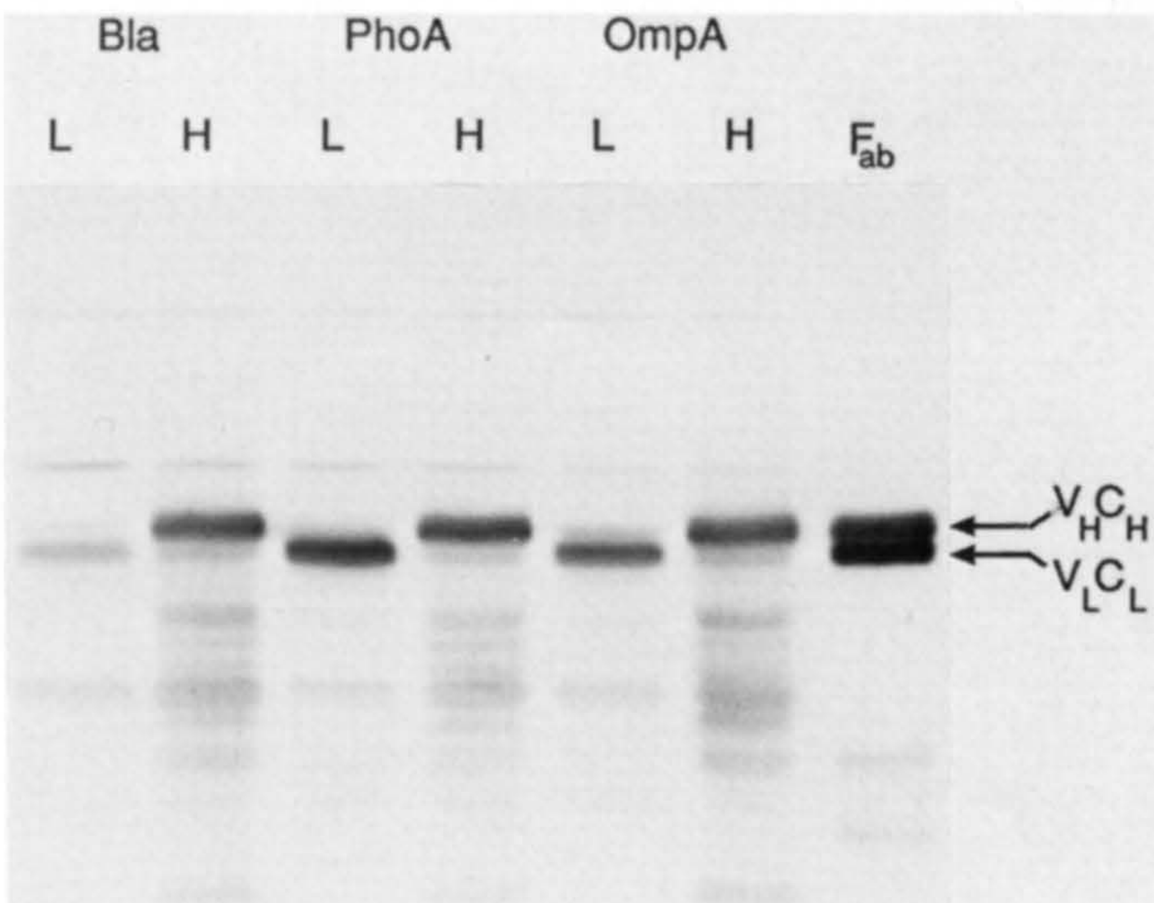

Fig. 3. Expression of individual light (V_LC_L, κ) and heavy (V_HC_H, F_d) chains of the F_{ab} fragment in precise fusions with the signal sequences of β-lactamase (*Bla*), alkaline phosphatase (*PhoA*) and outer membrane protein A (*OmpA*). An aliquot of total protein from cells (corresponding to <0.4 ml·OD_{550}) was separated on a 14% polyacrylamide gel and detected by immunostaining after transfer to nitrocellulose. Purified F_{ab} fragment was also applied as a control. Both the techniques and the plasmids are described in Materials and methods.

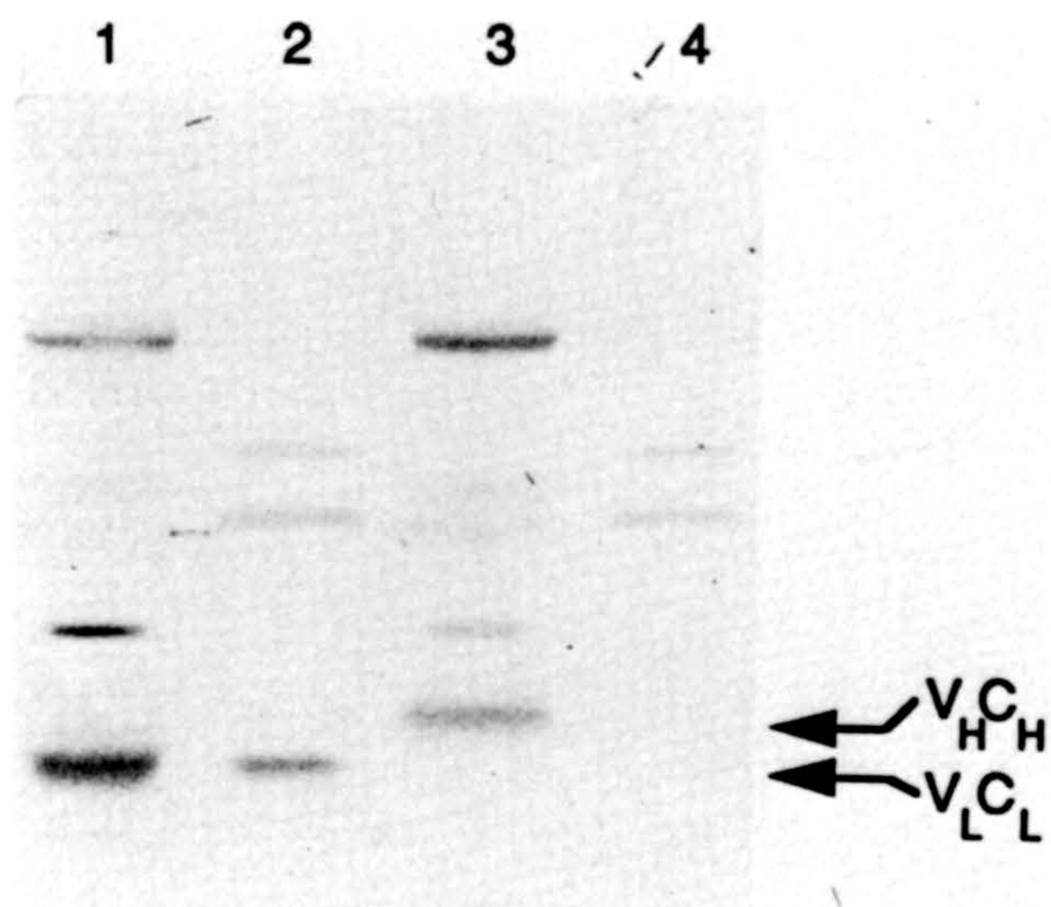

Fig 4. Cell fractionation of *E.coli* expressing either the light chain (κ) or the heavy chain (F_d) of the F_{ab} fragment, each fused precisely to the *phoA* signal sequence. The proteins were detected by immunostaining after SDS–PAGE on a 12.5% gel and transfer to nitrocellulose. The fractionation of the light chain construct (SM547/pASK7) is shown in **lanes 1** and **2**, whereas the heavy chain construct (SM547/pASK8) is shown in **lanes 3** and **4**. The spheroplast fractions are shown in **lanes 1** and **3**, and the periplasmic fractions in **lanes 2** and **4.**

with native heterodimers in the case of F_v and F_{ab} fragments. Slow folding steps would be expected to divert the desired folding pathway to the aggregation of incorrectly folded intermediates (Jaenicke, 1987). These aggregates may even become covalently linked by disulphide bonds in the oxidizing milieu of the periplasm. Using protein engineering, we therefore sought to identify folding steps of the antibody fragments that may occur slowly in *E.coli* and are likely to occur at different rates in the F_{ab} and F_v fragment. In light of the results described above we concentrated on structural factors located in the C_H1 domain of the F_{ab} fragment in our initial experiments.

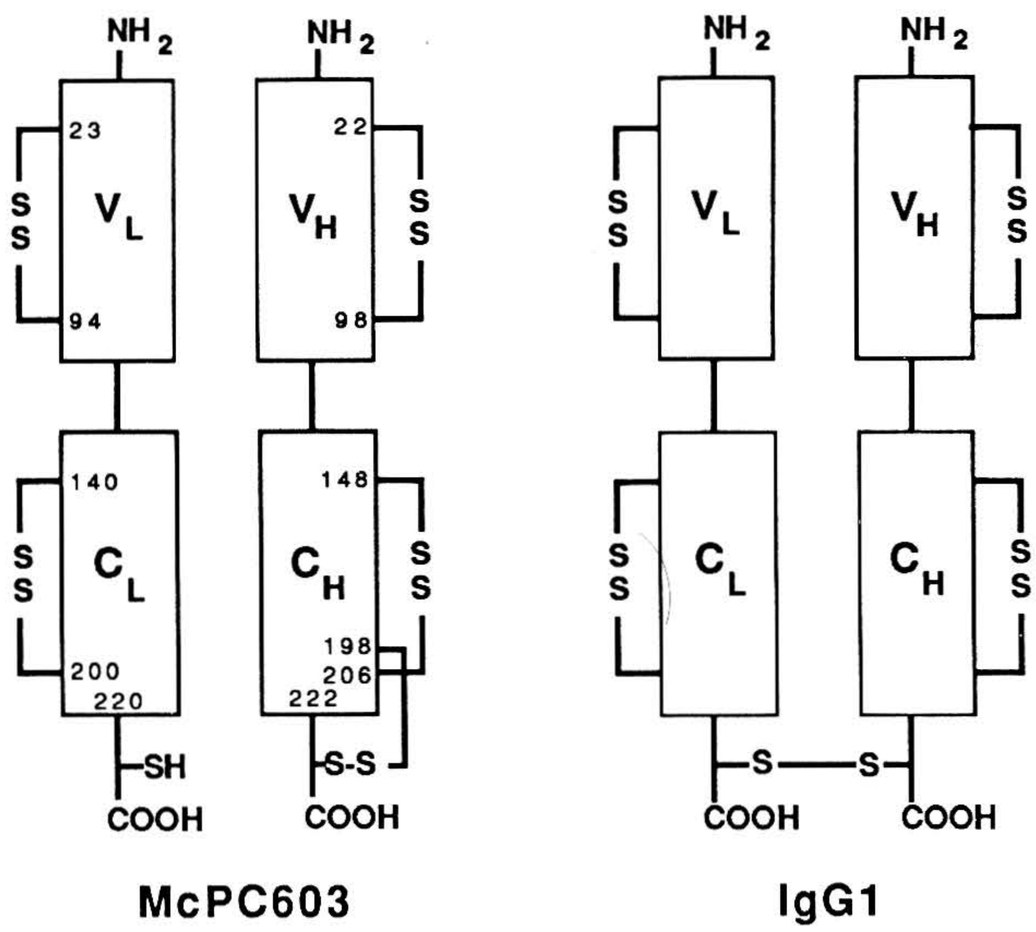

Fig. 5. The arrangement of the disulphide bonds in the F_{ab} fragment of McPC603, an IgA (**left**) and the *IgG1* variant (**right**). The numbers of the cysteine residues are shown in the left-hand scheme (numbering according to the sequence of McPC603).

Disulphide bonds

The first potentially slow process we considered is the formation and isomerization of disulphide bonds. The F_d fragment of McPC603 contains six cysteine residues and the $\varkappa$ light chain contains five cysteine residues, whereas both the V_H and the V_L domains possess only two. The arrangement of the disulphide bonds in the F_{ab} fragment is shown diagrammatically in Figure 5. Note that there is no covalent link between heavy and light chain in either the F_v fragment or in the F_{ab} fragment of McPC603. The presence of a larger number of cysteine residues in the F_{ab} fragment should give rise to a larger number of possible intramolecular crosslinks and therefore potentially to a larger number of disulphide rearrangement steps. This might conceivably lead to a slower and less efficient formation of the native disulphide bonds in the F_{ab} fragment compared with the F_v fragment.

Each antibody domain possesses a characteristic central disulphide bond (Williams and Barclay, 1988; Hunkapiller and Hood, 1989) linking the two layers of β-sheet. However, the C_H1 domain of mouse IgA contains an additional disulphide bond. It was therefore of interest to remove this disulphide bond between CysH198 and CysH222 by replacing both cysteine residues with alanine residues and to investigate the influence of this replacement on the yield of functional F_{ab} fragment. The removal of this disulphide bond still leads to a functional protein but does not cause a significant change in the functional expression yield of the F_{ab} fragment (Figure 6 and Table II). The total expression level was also essentially unchanged in this mutant as well as in all the other mutants discussed below, when compared with the wild-type F_{ab} fragment (data not shown). While the accuracy of the determinations of absolute amounts of protein is only $\sim \pm 50\%$, the conclusion that the fraction of correctly folded molecules changes only very marginally is very clear. This finding demonstrates that the disulphide bond between

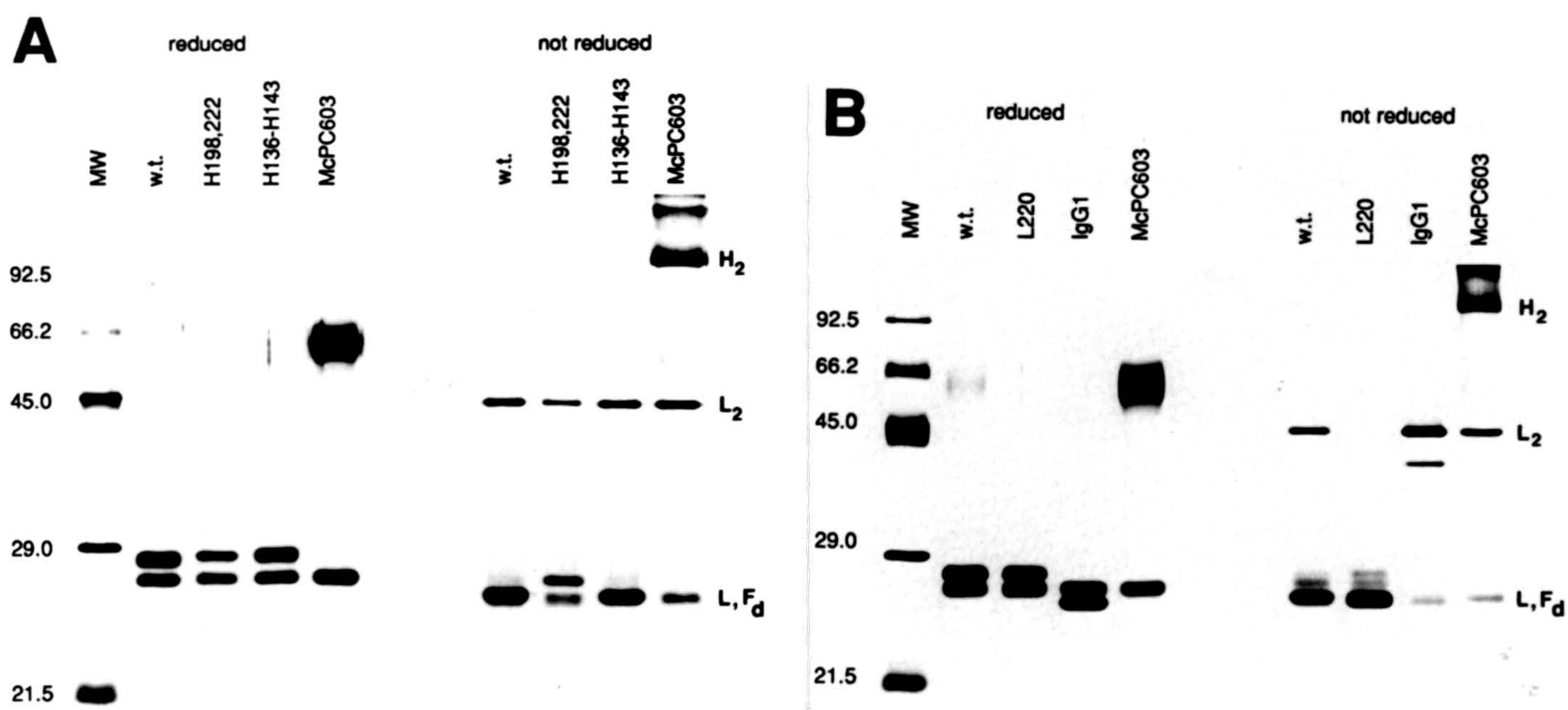

Fig. 6. SDS–PAGE (12.5%) of the variants of the F_{ab} fragment expressed in *E.coli* under non-reducing and reducing conditions detected by silver staining. The samples in the five left-hand lanes in (**A**) and (**B**) were reduced prior to electrophoresis as indicated, whereas the samples in the four right-hand lanes were not reduced. The fragments are the wild-type F_{ab} fragment (denoted w.t.), the variants *H198,222, H136-H143, L220*, the *IgG1* variant and the whole antibody (denoted McPC603) isolated from mouse ascites. The covalently linked F_{ab} fragment of the *IgG1* variant runs at about the same position as the L_2 dimer.

CysH198 and CysH222 is dispensable for folding and stability under these conditions, but also that its presence is unlikely to be the major cause for the low folding yield of the F_{ab} fragment.

In contrast to this result, the removal of another disulphide bond, between CysH22 and CysH98 within V_H (see Figure 5) prevented the formation of any functional F_{ab} fragment (mutant *H22,98*) that could be purified from the *E.coli* cells by hapten-affinity chromatography. This result is rather unexpected, since antibodies have been described and characterized previously (Rudikoff and Pumphrey, 1986), which naturally lack the disulphide bond in V_H.

In a third experiment on the effect of disulphide bond formation, CysL220 in the $\varkappa$ chain of the F_{ab} fragment was changed into alanine (mutant *L220*). This is the only cysteine residue of this antibody fragment which does not participate in an intramolecular disulphide bond. However, it can form an intermolecular disulphide bond leading to the covalent linking of the light chains and thus to the formation of dimeric F_{ab} fragments. This unusual phenomenon, which is caused by the lack of a free cysteine residue in the F_d chain and which has been noted before for BALB/c mouse IgA proteins (Abel and Grey, 1968; Seki *et al.*, 1968; Grey *et al.*, 1970; Nisonoff *et al.*, 1975), also occurs to some extent during the expression of the F_{ab} fragment of McPC603 in *E.coli* (see Figure 6). Yet, the removal of CysL220 did not significantly change the yield of functional recombinant F_{ab} fragment. It should be pointed out, however, that the chemically more homogeneous form of this protein might be advantageous for some applications.

Table II. Yield of correctly assembled F_{ab} fragment for the variants constructed

F_{ab} variant	Strain/plasmid	Yield of functional F_{ab} fragment[a] (mg/l/OD_{550})
Wild-type	JM83/pASK37	0.13
H136-H143	JM83/pASK37-*H136-H143*	0.36
H22,98	JM83/pASK37-*H22,98*	–
H198,222	JM83/pASK37-*H198,222*	0.15
L220	JM83/pASK37-*L220*	0.22
IgG1	JM83/pASK37-*IgG1*	0.31

[a]The experiments were carried out after growth at ambient temperature. The percentage of correctly assembled F_{ab} fragment compared to totally expressed F_{ab} fragment can be estimated from Table I.

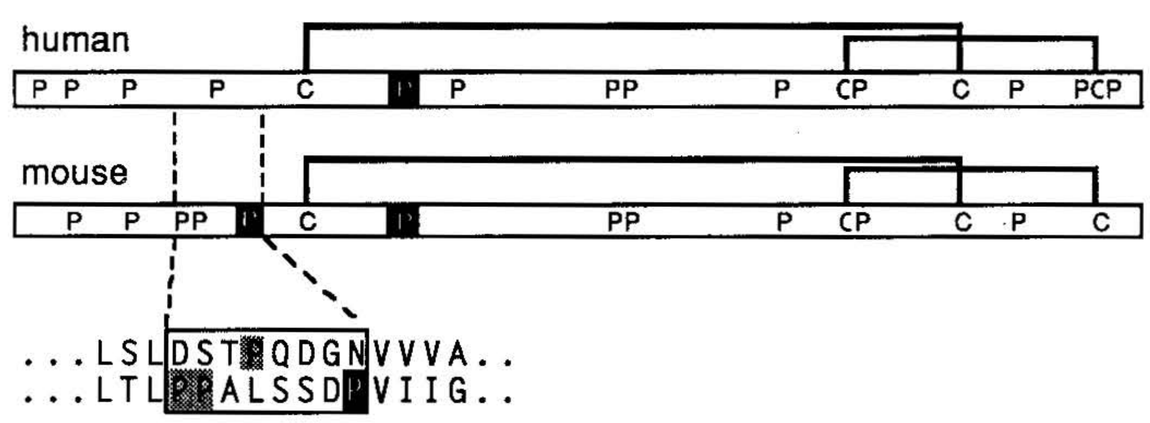

Fig. 7. Schematic arrangement of cysteines and prolines in the C_H1 domain of human and mouse IgA. The sequence around the region, where a loop was replaced, is shown. The exchanged piece of sequence is boxed, and the *cis*-proline sequences are emphasized white on black.

Disulphide analysis and functionality of the mutant F_{ab} fragments

The differences in the disulphide bond patterns between the mutants described above, the wild-type F_{ab} fragment and an *IgG1* variant (in which the C_H1 domain from IgG1 replaces the IgA domain; see below), can be assessed from a comparison of SDS–PAGE analyses under reducing and non-reducing conditions (Figure 6A and B). The reduced light chain has the same mobility in all recombinant proteins, as well as in the native antibody, as expected. The same is true under non-reducing conditions where the monomeric light chains have an enhanced mobility due to two intrachain disulphide bonds. However, in the native antibody McPC603, isolated from mouse ascites, dimeric light chains are observed in addition to the monomeric form under non-reducing conditions. All recombinant light chains also show partial dimerization with the exception of the mutant *L220*, where the C-terminal cysteine residue is missing, and of the *IgG1* variant (see below).

The reduced F_d chains all have identical mobilities except in the case of the *IgG1* variant where the F_d fragment migrates faster than the $\varkappa$ chain. The different mobility of the IgA F_d fragment, the *IgG1* F_d fragment and the $\varkappa$ chain under reducing conditions is remarkable as all three chains have very similar lengths (220 amino acids for the light chain, 222 for the $\varkappa$ IgA F_d and 224 amino acids for the *IgG1* F_d chain), corresponding to calculated mol. wts of 24.1, 24.3 and 24.2 kDa respectively.

Interestingly, the oxidized IgA F_d chains have mobilities which are identical to those of the oxidized monomeric light chains. One exception is the mutant, in which the disulphide bond H198–H222 was removed, and which migrates distinctly more slowly. Since the absence of one disulphide bond is unambiguously detectable by a lower mobility under non-reducing conditions, this observation implies that in the wild-type fragment and in the other mutants discussed all disulphide bonds have been formed quantitatively. It may be noted in this context that Cockle and Young (1985) suggested that the formation of the central disulphide bonds is not quantitative in the C_H1 domain of natural mouse IgA. A second exception showing a different mobility of the F_d fragment under non-reducing conditions compared with the wild-type is the *IgG1* variant. In this case, both chains of the F_{ab} fragment are covalently linked by a disulphide bond as is typical for the IgG1 class.

All fragments with the exception of *H22,98* bound to the hapten-affinity column. In order to quantitatively test the functionality of the *IgG1* variant of the McPC603 F_{ab} fragment,

Table III. Comparison of loop from human IgA to other proteins in the Brookhaven Data Base

Begin. residue[a]	Sequence	PDB entry	Protein	Reference
H136	D S T P Q D G N	–	Human IgA	Flanagan *et al.* (1984)
115	A S G P N D G L	2APR	Acid proteinase	Suguna *et al.* (1987)
203	D S H P A D G I	3CNA	Concanavalin A	Hardman and Ainsworth (1972)
135	D T T P D N G R	2CYP	Cytochrome *c* peroxidase	Finzel *et al.* (1984)
92	L F T P Q C G K	5ADH	Alcohol dehydrogenase	Eklund *et al.* (1984)
17	D G D P D D G V	4RXN	Rubredoxin	Watenpaugh *et al.* (1980)

[a]The residue number with which the piece of sequence shown begins. The residue number given for IgA is the number in the sequence of McPC603, where this loop was inserted. Residues identical as in the human IgA loop are underlined.

we also determined the binding constant for the hapten phosphorylcholine by fluorescence titration (Glockshuber *et al.*, 1990) for this protein. The value of 1.80×10^5 M obtained was essentially identical to that of the wild-type F_{ab} fragment prepared by proteolysis or purified as a recombinant protein from *E.coli* (Skerra *et al.*, 1990).

Proline cis–trans isomerization

Another slow folding step that has been identified from *in vitro* refolding experiments is the isomerization of the peptide bond N-terminal to proline residues (Brandts *et al.*, 1975; Schmid and Baldwin, 1978; Lang *et al.*, 1987; Lin *et al.*, 1988; Fischer *et al.*, 1989; Fischer and Schmid, 1990). The crystal structure of McPC603 shows that the protein contains five *cis*-proline residues (L8 and L101 in V_L, L147 in C_L and H143 and H155 in C_H1). In order to investigate the role of proline *cis–trans* isomerization for the functional expression of the F_{ab} fragment by protein engineering, an important aspect has to be taken into consideration. If such a *cis*-proline residue were simply replaced by a different amino acid residue, the protein would very likely be destabilized, as the *cis*-peptide bond is probably a consequence of the local structural requirements and other *cis*-peptide bonds are much less likely to form (Ramachandran and Mitra, 1976; Stewart *et al.*, 1990). Thus, we chose a structurally less perturbing approach.

A comparison of the sequences of human and mouse IgA C_H1 domains showed that one loop of McPC603, containing the *cis*-proline residue H143 and two proline residues with *trans* conformation, corresponds to a completely different sequence in the human IgA2m(1) allotype (Figure 7) (Flanagan *et al.*, 1984). In contrast, the proline at position H155 is conserved. The loop around ProH143 is located at one end of the C_H1 domain facing away from the variable domain. As the regions adjacent to the loop are well conserved between the IgAs from mouse and man, we decided to replace the whole mouse loop of eight amino acids with the human sequence as shown in Figure 7. From X-ray studies of proteins containing homologous sequences (Table III) and from statistical evaluations (Frömmel and Preissner, 1990), it can be postulated that the peptide bond of the single proline residue occurring in the human sequence is likely to be *trans*, although direct experimental evidence in this particular case is lacking.

The resultant mutant F_{ab} fragment *H136-143* was expressed and purified by hapten-affinity chromatography in the same way as the recombinant wild-type F_{ab} fragment (Figure 6). However, the loop replacement led only to a rather modest increase in the yield of correctly folded antibody fragment (Table II). This shows that proline *cis–trans* isomerization is unlikely to be the most decisive factor for the limitation of the folding yield of the F_{ab} fragment in *E.coli*.

An artificial class switch

We finally changed the whole mouse C_H1 domain in the F_{ab} fragment from class IgA to IgG1. There are several structural features affected by this substitution. First, the C_H1 domain from an IgG1 forms an interchain disulphide bond to C_L with its C-terminal cysteine residue as shown in Figure 5. Second, the C_H1 domain from an IgG1 possesses only the single central intradomain disulphide bond (see Figure 5). Third, in contrast to the C_H1 domain of McPC603, the C_H1 domain from an IgG1 is not glycosylated in the native antibody (summarized in Nisonoff *et al.*, 1975). While we showed recently that this glycosylation in C_H1 of McPC603 does not affect antigen affinity (Skerra *et al.*, 1990), it might still affect the efficiency of folding *in vivo* (Taylor and Wall, 1988). The *IgG1* variant of the McPC603 F_{ab} fragment was purified in functional form by hapten-affinity chromatography (Figure 6) in a yield that appeared to be only marginally higher than for the original recombinant F_{ab} fragment (Table II).

Discussion

What limits the yield of the functional protein?

Our results show that a number of variants in the constant domains of the F_{ab} fragment of the antibody McPC603 can be functionally expressed in *E.coli* and purified to homogeneity in a single step by hapten-affinity chromatography. The choice of the signal sequence used for the secretion of the light and heavy chain of the F_{ab} fragment in *E.coli* is not critical and seems to be rather flexible. In the case of the antibody McPC603, the *E.coli* signal peptides of the Bla, OmpA and PhoA proteins were found to be fully interchangeable and other F_{ab} fragments have been successfully secreted in *E.coli* using the *pel*B leader peptide from *Erwinia carotovora* (Better *et al.*, 1988; Huse *et al.*, 1989). In contrast, the heavy chain of the McPC603 F_{ab} fragment is not correctly secreted with its original eukaryotic signal sequence in *E.coli*. A possible explanation for this finding is the fact that the last amino acid in this signal sequence is a cysteine residue (Kim *et al.*, 1981), which does not seem to occur in *E.coli* signals (Watson, 1984; Sjöström *et al.*, 1987). It thus seems possible that the expression of this particular preprotein leads to the inhibition of the *E.coli* signal peptidase I which would account for the severe toxic effect observed in this case. A similar phenotype has been observed when a proline residue follows the signal peptidase cleavage site (Plückthun and Knowles, 1987), and may be only poorly cleaved.

The quantitative analysis of the functional expression of the McPC603 F_{ab} fragment in *E.coli* shows that a significantly smaller fraction is assembled to the functional heterodimer than is found for the F_v fragment. The limiting process does not seem to be expression, secretion or processing, which are all found to be similar to the F_v fragment, but rather folding and assembly of the recombinant protein. Thus, the question arises which factors are responsible for the undesired fate of the main portion of the expressed F_{ab} fragment.

Interestingly, the absolute yield of functionally expressed F_{ab} fragment remains constant with increase of the total expression level in *E.coli* (Table I) and the fraction of functional protein consequently decreases. A similar phenomenon has been described by, for example, Takagi *et al.* (1988) in the case of the secretion of subtilisin E in *E.coli*. However, it is unlikely that the high expression level of the secreted recombinant F_{ab} fragment achieved here is *per se* the cause for the observed phenomenon, since the F_v fragment and the F_{ab} fragment are expressed at a very similar level of total protein. Furthermore, several eukaryotic proteins were shown to be secreted into the periplasm of *E.coli* in higher yields of functional protein, some of them with multiple disulphide bonds or even an immunoglobulin fold (Hsiung *et al.*, 1986; Chang *et al.*, 1987; Takahara *et al.*, 1988; Dalboge *et al.*, 1989). Recently, it could be shown (Holmgren and Bränden, 1989) that even certain *E.coli* strains themselves express a periplasmic protein (named PapD) with immunoglobulin topology. Thus it seems unlikely that the periplasmic space of *E.coli* provides an environment generally unsuitable for the folding of the F_{ab} fragment. It has to be asked, therefore, whether specific structural parameters can be identified for this protein which limit the folding yield of the F_{ab} fragment when it is expressed in *E.coli*.

Recent experiments (Skerra *et al.*, 1990) demonstrated that the F_{ab} fragment of McPC603 is also glycosylated in the C_H1

domain, consistent with an earlier proposal from additional electron density in its X-ray structure (Satow *et al.*, 1986). However, the absence of glycosylation is unlikely to be the reason for the low-folding yield, since the *IgG1* variant of the F_{ab} fragment possesses a C_H1 domain that is not glycosylated in the animal either. The folding yield of this protein is only insignificantly higher in *E.coli* than that of the wild-type F_{ab} fragment.

Processed, but insoluble forms of eukaryotic proteins secreted in *E.coli* have been observed before (Libby *et al.*, 1987; Wong *et al.*, 1988) and even overexpressed *E.coli* RTEM β-lactamase has been found to form periplasmic inclusion bodies of the mature protein (Bowden *et al.*, 1991) or cytoplasmic inclusion bodies of the precursor (Laminet and Plückthun, 1989). Yet in no protein has the structural factor limiting the folding yield been elucidated. Thus, we investigated this phenomenon with a series of mutants of the F_{ab} fragment.

The role of proline cis–trans isomerization

In order to narrow down distinct structural features of the F_{ab} fragment that may cause the limitation of the folding yield we focused our attention first on the role of proline *cis–trans* isomerization. This reaction has been identified as a slow step in protein folding (Brandts *et al.*, 1975; Schmid and Baldwin, 1978; Fischer and Schmid, 1990) and it is conceivable that an intermediate not having undergone this isomerization might accumulate, aggregate, and thus be diverted from the desired folding pathway. Experiments with thioredoxin have demonstrated that the removal of the *cis*-proline residue can indeed remove the slow phase in an *in vitro* folding assay (Kelley and Richards, 1987).

We changed a whole loop, in which eight amino acids including two *trans*-proline residues and one *cis*-proline from the mouse IgA C_H1 domain were replaced by a loop from the human IgA2m(1) allotype containing only one proline residue, most likely in *trans* configuration (referenced in Table III). The increase in the folding yield of the mutant F_{ab} fragment by a factor of 2–3, as a consequence of this mutation, is consistent with the influence of the *cis–trans* isomerization of ProH143. Yet, the folding yield of this mutant still only amounts to a modest fraction of the total expressed antibody protein. Since the heavy chain has only two *cis*-proline residues, the effect is thus too small to be solely responsible for the observed lower folding yield of the McPC603 F_{ab} fragment compared with the F_v fragment.

The importance of the possible catalysis of proline *cis–trans* isomerization *in vivo* remains unclear (Fischer and Schmid, 1990). While even *E.coli* possesses a peptidyl prolyl-*cis–trans* isomerase (Liu and Walsh 1990), its role in protein folding *in vivo* remains to be elucidated. It does remain conceivable, however, that this step is less efficiently catalysed for some foreign protein in *E.coli* than in the eukaryotic cell.

The role of disulphide isomerization

Another process important in folding that we scrutinized is the formation and isomerization of disulphide bonds. *In vitro* experiments have suggested the kinetic significance of this process in some proteins (Creighton, 1978). In particular, pioneering studies on bovine pancreatic trypsin inhibitor (Creighton, 1978) and mutants of this protein (Marks *et al.*, 1987) appeared to be consistent with a disulphide rearrangement being a mandatory step in the *in vitro* folding of this protein. Yet, these results critically depend on the methodology used for detecting intermediates and have now been challenged (Weissman and Kim, 1991).

The situation *in vivo* is even less clear. Bergman and Kuehl (1978, 1979) showed that the intra-domain disulphide bonds of an immunoglobulin light chain form co-translationally in a sequential manner. Nevertheless, the presence of two disulphide bonds within the same domain, as in the case of the C_H1 domain of the McPC603 F_{ab} fragment, might lead to a kinetically significant isomerization process. The lack of the C-terminal part of the complete heavy chain is likely to cause an even more complex mechanism of disulphide bond formation in the case of an F_{ab} fragment, because the two disulphide bridges overlapping the sequence of the C_H1 domain can only form after the F_{ab} fragment has been released from the membrane. In contrast, the disulphide formation in the C_H1 domain in the complete heavy chain would be expected to occur while this chain is still bound to the membrane; some incorrect disulphide formations with other parts of the protein might thus be prevented.

However, the elimination of the second disulphide bond in the C_H1 domain (either by site-directed mutagenesis or by artificial class switching of the whole antibody domain) does not lead to a significant increase in the folding yield. Therefore, it seems unlikely that these processes are solely responsible for the folding problem of the F_{ab} fragment when expressed in *E.coli*. In addition, the removal of a C-terminal cysteine residue which does not participate in an intra-protein disulphide bond (in the mutant *L220*) also does not lead to a significant increase in the amount of functional protein.

This finding must be contrasted to experimental evidence that has been accumulated (Freedman, 1984, 1987, 1989; Freedman and Hillson, 1980) pointing to the importance of the catalysis of this process by protein disulphide isomerase in eukaryotic cells. Recently, a protein disulphide isomerase has also been discovered in the periplasm of *E.coli* (J.C.A.Bardwell, K.McGovern and J.Beckwith, in preparation; S.Kamitani, Y.Akiyama and K.Ito, in preparation), but its substrate specificity and precise mechanistic role remain to be elucidated.

Conclusions

We demonstrated that a wide variety of signal sequences leads to the functional expression and secretion of antibody F_{ab} fragments. Some leakage of the outer membrane is observed especially at higher temperatures, independent of the choice of signal sequence. This is not an indication for a specific 'secretion to the medium'.

Our protein-engineering experiments show that the structural features frequently suspected as influencing the yield of folding *in vivo* (proline *cis–trans* isomerization and disulphide isomerization) have by themselves only small effects on the partitioning of the folding pathway to the native structure *in vivo*.

It may thus be a rather general property of the immunoglobulin F_{ab} fragment rather than a particular structural feature which is responsible for the limitation of the folding yield in *E.coli*. In this respect, the emerging physiological role of other potential folding modulators, in addition to those already mentioned (i.e. proline *cis–trans* isomerase and protein disulphide isomerase) must be considered. The immunoglobulin heavy chain-binding protein (BiP), a 'molecular chaperone' (Ellis, 1987), seems to play a role in the association of antibody light and heavy chains *in vivo* (Haas and Wabl, 1983; Bole *et al.*, 1986; Hendershot *et al.*, 1987; Kassenbrock *et al.*, 1988; Nakaki *et al.*, 1989). The major role of such folding modulators may be the prevention of aggregation of folding intermediates, rather than any catalysis of folding itself. Suitable chaperones for larger antibody fragments in *E.coli* may be missing, may have an unsuitable specificity or may only be present in much too low amounts in the periplasm of the *E.coli* cell. The co-expression of several

molecular chaperones is now being investigated to determine the sensitivity of various fragments to this kind of catalysis. Furthermore, the investigation of homologous frameworks from different species may clarify some of the correlations between sequence and *in vivo* folding.

The facile quantitation of the yield of functional F_{ab} fragment in *E.coli* and the potential application even of functional assays on bacterial colonies makes this experimental system particularly useful for the future search for and identification of specific protein-folding catalysts.

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